TRADE SECRET

STUDY TITLE: H-28072: *In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster Ovary Cells

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines, OPPTS 870.5375 (1998)

OECD Guidelines for the Testing of Chemicals, Number 473 (1998)

EC Commission Directive 2000/32/EC Annex 4A-B10 Number L 136 (2000)

AUTHOR: Christine M. Glatt, M.S.

ORIGINAL REPORT COMPLETED: July 25, 2007

REPORT REVISION 1 COMPLETED: September 23, 2009

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
DuPont Haskell Global Centers for
Health & Environmental Sciences
P.O. Box 50
Newark, Delaware 19714
U.S.A.

LABORATORY PROJECT ID: DuPont-22620

WORK REQUEST NUMBER: 17199

SERVICE CODE NUMBER: 531

SPONSOR: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are compatible with current OECD Good Laboratory Practices, except for the items documented below. None of the items listed impact the validity of the study.

1. The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was not performed under Good Laboratory Practice Standards, the accuracy of the data is considered sufficient for the purposes of this study.

2. Neither the vehicle nor the positive controls were characterized by the testing facility or the sponsor. However, both the vehicle and positive controls were purchased from a reputable vendor and showed results consistent with historical control data.

3. The concentrations of the positive control and test substance dose solutions were not confirmed analytically; however, the solutions were prepared by trained personnel to ensure the accuracy of the concentrations.

Study Director: Christine M. Glatt   23-Sep-2009

Christine M. Glatt, M.S.
Senior Staff Toxicologist
Date

QUALITY ASSURANCE STATEMENT

Work Request Number: 17199
Service Code Number: 531

Key inspections for DuPont work request 17199, service code 531 were performed for the tasks completed at DuPont by the Quality Assurance Unit of DuPont and the findings were submitted on the following dates.

Phase Audited	***Audit Dates***	***Date Reported to Study Director***	***Date Reported to Management***
Protocol:	March 19, 2007	March 19, 2007	March 19, 2007
Conduct:	March 29, 2007	March 29, 2007	March 29, 2007
Report/Records:	May 29-30, 2007 July 16, 2007 July 24, 2007	May 30, 2007 July 16, 2007 July 24, 2007	June 7, 2007 July 16, 2007 July 24, 2007
Report Revision 1:	September 23, 2009	September 23, 2009	September 23, 2009

Reported by: Donna M. Johnston 23 Sep. 2009

Donna M. Johnston
Quality Assurance Auditor

Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Reviewed and Approved by: E. Maria Donner
E. Maria Donner, Ph.D.
Senior Research Toxicologist and Manager

23-SEP-2009
Date

Issued by Study Director: Christine M. Glatt
Christine M. Glatt, M.S.
Senior Staff Toxicologist

23-Sep-2009
Date

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

STUDY INFORMATION

Substance Tested:	• HFPO Dimer Acid Ammonium Salt • 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionic acid, ammonium salt • 62037-80-3 (CAS Number) • H-28072
Haskell Number:	28072
Composition:	82.6% Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionate* 13.9% Water 3.5% Ammonium 0.41% Organic Impurities * Note: The Ammonium-2,3,3,3-tetrafuoro-2-(heptafluoropropoxy) propionate component (HFPO Dimer ammonium salt) contains 0.1 ppm HFPO trimer ammonium salt.
Purity:	See composition, above
Physical Characteristics:	Clear and colorless concentrated aqueous solution
Stability:	The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.
Study Initiated/Completed:	March 15, 2007 / (see report cover page)
Experimental Start/Termination:	March 19, 2007 / April 30, 2007

REASON FOR REVISION 1

This report was revised to correct a typographical error on the units of the actual stock concentration listed in Results and Discussion section A. Solubility.

SUMMARY

The test substance, H-28072 was evaluated for its ability to induce chromosome aberrations *in vitro* in Chinese hamster ovary (CHO) cells in both the absence and presence of an exogenous S9 metabolic activation system (Aroclor-induced rat liver S9). Numerical aberrations were also recorded. To establish a concentration range for the chromosome aberration assay, a preliminary toxicity assay was initially conducted.

The test substance was prepared in water as this vehicle was determined to be the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance was soluble in the vehicle at 34.71 mg/mL (0.1M), the highest stock concentration that was prepared for use on this study. The test substance formed a clear solution in water at the highest prepared stock concentration.

In the preliminary toxicity assay, the cells were treated for 4 and 20 hours in the non-activated test conditions and for 4 hours in the S9-activated test condition. All cells were harvested 20 hours after treatment initiation. A vehicle control group was included in each test condition.

In the preliminary toxicity assay, the highest concentration tested was 3471 µg/mL (10 mM). Due to the discrepancy between the initial sponsor-reported and COA-reported purities, the actual maximum exposure concentration tested was 3391 µg/mL. The lower concentrations were consistently reduced by 2.3% each. All subsequent exposure concentration levels in this report represent the nominal values based in the initial sponsor-reported purity (84.5%) used for study conduct. The cells were exposed to seven concentrations of the test substance ranging from 50 to 3471 µg/mL, as well as positive controls and a vehicle control. No visible precipitate was observed in the treatment medium at the beginning or end of the treatment period at any concentration tested. The osmolality of the highest test substance concentration in medium was similar to the vehicle control both in the absence and presence of S9. The pH of the highest test substance concentration in medium was similar to the vehicle control in both the absence and presence of S9. However, a slight color change of the media at concentrations ≥2000 µg/mL were noticed at the beginning and end of the treatment periods possibly representing a slight increase in pH.

Substantial toxicity (greater than a 50% reduction in cell growth relative to the vehicle control) was observed at 3471 µg/mL in the 20-hour non-activated test condition only. The mitotic inhibition in the 20-hour non-activated test condition at 3471 µg/mL was 96%. Target concentrations for the chromosome aberration assay were achieved in the preliminary toxicity assay. Slides were coded and evaluated for clastogenicity and no separate chromosome aberration assay was conducted. Selection of doses for microscopic analysis was therefore based on these dose concentration levels.

Cytogenetic evaluations were conducted at 1000, 2000, and 3471 µg/mL for the 4-hour non-activated and activated test conditions and 500, 1000, and 2000 (5.76 mM) µg/mL for the 20-hour non-activated test condition. These concentrations were chosen based on the toxicity data and scorability of the slides (i.e., metaphase quality, chromosome morphology, and a sufficient amount of metaphases present). The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control at any concentration ($p \geq 0.05$, Fisher's exact test). However, in the 4-hour activated test condition, the control value for numerical aberrations was high (outside of the historical control range).

A confirmatory trial (trial 2) was conducted at concentration levels of 250, 500, 1000, 2000, and 3471 µg/mL for the 4-hour activated test condition to confirm the results initially obtained in trial 1 (preliminary toxicity assay) for this test condition. No visible precipitate was observed in the treatment medium at the beginning or end of the treatment period at any concentration tested. No substantial toxicity was observed at any concentration level. Cytogenetic evaluations were conducted at 1000, 2000, and 3471 µg/mL. The percentage of cells with structural aberrations was significantly increased above that of the vehicle control at 3471 µg/mL ($p < 0.05$, Fisher's exact test). The response was outside the historical control range and is considered biologically significant.

All criteria for a valid study were met. Under the conditions of this study, H-28072 was found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells in the S9-activated test system. It was concluded that the test substance was positive in this *in vitro* test.

INTRODUCTION

The objectives of this study were to evaluate the test substance, H-28072, for its ability to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. Numerical aberrations were recorded. The assessment was done both in the presence and absence of an exogenous S9 metabolic activation system.

MATERIALS AND METHODS

A. Test Guidelines

Except as noted below, the study design complied with the following test guidelines:

- U.S. Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870.5375, *In Vitro* Mammalian Chromosome Aberration Test. (August, 1998).
- Ninth Addendum to the OECD (Organisation for Economic Cooperation and Development) Guidelines for the Testing of Chemicals, *In Vitro* Mammalian Chromosome Aberration Test, No. 473. (February, 1998)
- European Commission Directive 2000/32/EC of May 19, 2000, Annex 4A-B10. Mutagenicity - *In Vitro* Mammalian Chromosome Aberration Test. No. L 136.

– The initial sponsor-reported purity for H-28072 was 84.5% active ingredient. A correction factor of 1.183 was used for preparation of the dosing solutions. However, the COA that was issued after the experimental termination of the study reported a purity of 82.6%. The guideline recommended limit dose for this test system is 10 mM (3471 µg/mL). Although the actual maximum dose (3391 µg/mL) did not reach this limit, the difference (2.3%) was considered negligible. This deviation did not impact the validity or outcome of the study.

B. Test Substance and Controls

1. Identification

The test substance, H-28072, was a clear and colorless concentrated aqueous solution. The test substance used for this study was assigned Haskell identification number 28072. Additional information regarding the test substance is located on the study information page of this report.

2. Characterization

The test substance was characterized by the sponsor prior to this study. The Certificate of Analysis (COA) of the test substance is included in this report (Appendix A).

3. Sample Preparation, Stability, and Analytical Verification of Test Substance Concentrations

The initial sponsor-reported purity for H-28072 was 84.5% active ingredient. A correction factor of 1.183 was used for preparation of the dosing solutions. However, the COA that was issued after the experimental termination of the study reported a purity of 82.6%. An analytical verification of the test substance concentrations was not conducted.

4. Controls

Negative:	Sterile Water (CAS# 7732-18-5, molecular grade, Mediatech Inc.)
Positive:	Mitomycin C (MMC, CAS# 50-07-7, Sigma) Cyclophosphamide (CP, CAS# 6055-19-2, Sigma)

The positive controls were dissolved in sterile water. The positive controls were assumed to be stable during this assay and no evidence of instability was observed.

C. Test System

The CHO-K_1 cell line was originally derived as a subclone from a parental CHO cell line. The cells require proline in the medium for growth, and have a modal chromosome number of 20. The population doubling time is 10-14 hours. The cell line was obtained from the American Type Culture Collection (ATCC number CCL 61), Manassas, Virginia. The karyotype and the absence of mycoplasm infection are routinely checked by Haskell Laboratory. This test system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.[(1)]

D. Experimental Design and Methodology

The study was conducted according to published procedures.[(1-5)] The test substance, as well as positive and negative (vehicle) controls, was administered in the presence and absence of an exogenous S9 metabolic activation system to cell cultures by addition to the culture medium. In the non-activated test system, the treatment times were approximately 4 and 20 hours, and in the S9-activated test system, approximately 4 hours. The dividing cells were arrested in metaphase approximately 18 hours after initiation of the treatment and harvested at approximately 20 hours. This harvest timepoint represents approximately 1.5 normal cell cycles, and is determined to ensure assessment of clastogenicity in first-division metaphase cells.[(4)] Cytogenetic analyses were conducted on the 4- and 20-hour non-activated and 4-hour activated assays. If a positive response was observed in the 4-hour non-activated assay, the 20-hour non-activated assay may not be scored. The cytogenetic assessment also included recording of numerical aberrations. Based on OECD 473, a clear positive response does not require verification. Negative results do not require confirmation, but are justified. Equivocal results may need to be confirmed, and may require a modified study design.

1. Solubility Determination and Selection of Vehicle

A solubility determination was conducted to determine the maximum soluble concentration of a workable suspension up to a maximum of 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Vehicles compatible with this test system, in order of preference, included, but were not limited to, culture medium or sterile water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), or ethanol (CAS 64-17-5). The vehicle of choice for this study was water, which permitted preparation of the highest workable/soluble stock concentration. Under the conditions of this test system, the concentration of water or physiological buffers did not exceed 10% of the treatment medium. After the addition of the dosing solution, the treatment medium was observed for precipitation (with the naked eye).

2. Exogenous Metabolic Activation

Liver homogenate (S9), prepared from male Sprague-Dawley rats induced with Aroclor 1254, were purchased commercially (Moltox, Inc., Boone, NC) and stored frozen at approximately -70°C until used. The protein concentration was 43.2 mg/mL for trial 1 and 43.3 mg/mL for trial 2 as reported by the vendor.

Immediately prior to use, the S9 liver homogenate was thawed and mixed with a cofactor pool. The final concentration of the cofactors and S9 in the metabolic activation system (S9 mixture) was 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer, pH 7.4, and 10% S9. The S9 mixture was prepared immediately before use and kept on ice until used. The metabolic activity of the S9 was demonstrated by the response of the CP-treated cultures.

3. Flask Identification

Using computer generated labels or a permanent marker, each flask or tube was labeled with the work request number, the Haskell number, dose level, replicate indicator (A or B), metabolic activation system (+/-S9), exposure period, and date.

4. Frequency and Route of Administration of the Dosing Solutions

Cell cultures were treated once for approximately 4 hours in the absence and presence of S9 metabolic activation, and for 20 hours in the absence of metabolic activation. The test substance was added to the treatment medium in a test system-compatible vehicle. This frequency and route of administration has been demonstrated to be effective in the detection of chemically-induced mutagenesis in this test system.[2-3]

5. Preparation of Target Cells for the Preliminary Toxicity Assay

Exponentially growing CHO-K_1 cells were seeded in labeled, sterile flasks. Approximately 5×10^5 cells/25 cm^2 flask were inoculated in complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units penicillin/mL and 100 µg streptomycin/mL). Cultures were incubated at 37 ± 2°C in a humidified atmosphere of 5 ± 2% CO_2 in air for 16-24 hours.

6. Preliminary Toxicity Assay to Select Dose Levels

a. Negative Control

The test substance vehicle was used as the concurrent negative control. The final concentration of water in the treatment medium did not exceed 10%.

b. Positive Control

The positive control substances were mitomycin-C (MMC) for the non-activated system and cyclophosphamide (CP) for the S9-activated system. The concentrations for MMC were 0.2 and 0.4 µg/mL, and for CP, 5 and 10 µg/mL. Both positive control substances were dissolved in sterile water. Two test concentrations of each positive control substance were used to ensure a valid assay; however, only one of the concentrations of each positive control was included in the cytogenetic analysis. The exposure periods for MMC were approximately 4 and 20 hours, and the exposure period for CP was approximately 4 hours. Exposure to the positive control substances was included in the chromosome aberration assay, but not in the preliminary cytotoxicity portion of the study. The positive control were not expected to contain any contaminants that would interfere with the conduct of the study, and were expected to be was stable under the conditions of administration. Concentration verification of the positive control substances was not conducted. No positive control substances were used for numerical aberrations (polyploidy or endoreduplication, or both).

c. Treatment of Target Cells

The day when the cells were first exposed to the test substance was designated as test day 0. Approximately 16-24 hours after seeding the CHO cultures, the culture medium was discarded and replaced with approximately 5 mL complete medium for the non-activated test condition, and 4 mL complete medium plus 1 mL of the S9 mixture for the activated test condition. The volumes were selected such that addition of the test substance volume (50 µL or 0.5 mL depending on the solvent) resulted in a total volume of approximately 5 mL.

Sets of duplicate cultures were then administered an aliquot of the test substance (seven concentrations were applied), the vehicle control, or two positive control substance concentrations for each test condition. The treatment medium was McCoy's medium for all test conditions. The cells were treated for approximately 4 and 20 hours in the non-activated test conditions, and for approximately 4 hours in the S9-activated test condition. After completion of the 4-hour exposure periods only, the cells were collected by centrifugation, washed once with phosphate buffered saline, fed with complete medium, and incubated until cell harvest. The incubations were conducted at 37 ± 2°C in a humidified atmosphere of 5 ± 2% CO_2 in air.

The osmolality and pH of the vehicle control, as well as the highest soluble test substance concentration in the culture media, were determined. An osmolality increase of ≤20% in the test substance treatment medium relative to the vehicle control treatment medium was considered acceptable. The pH of the treatment medium was evaluated both at the beginning and end of the treatment period by visual determination using the pH-sensitive color dye present in the treatment medium. If necessary, the pH of the treatment medium was adjusted to maintain a neutral pH based on visual inspection. Documentation was made in the study records. A visual inspection of the treatment medium for precipitation was also conducted at the beginning and the end of the treatment period.

d. Collection of Metaphase Cells and Cell Harvest

The cells were arrested in metaphase approximately 18 hours after treatment initiation by adding Colcemid® to the cultures at a 0.1 µg/mL final concentration in the culture media. Approximately 20 hours after treatment initiation, the cultures were washed with PBS, trypsinized, collected by centrifugation, and the cells were resuspended in 5 mL fresh medium. A concurrent cytotoxicity measurement determining total cell growth inhibition (%) relative to the solvent control was conducted for all assays and test conditions using an automated cell counter. In addition, a physical examination of cell growth (monolayer cell confluency) was conducted for all test conditions. The cells were treated with 0.075M KCl hypotonic buffer, fixed once in methanol and 3 times in methanol:glacial acetic acid (3:1 v/v), and stored frozen. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. One slide per culture was prepared by applying an aliquot of the fixed cells onto clean microscope slides and air-drying them. The slides were stained by Giemsa and permanently mounted.

permanently mounted.

e. Identification of the Slides

The slides were identified by the work request number, the Haskell number, dose level, replicate indicator (if applicable, i.e., A, B, C, etc.), metabolic activation system (+/-S9), exposure period (4 or 20 hours), and date.

f. Cytogenetic Analyses

For each test condition, cytogenetic analyses were conducted for at least three test substance concentrations, the vehicle control, and a positive control. The highest test substance concentration level that was analyzed was that which induced a greater than 50% cell growth inhibition relative to the vehicle control or, if the test substance lacked toxicity, the highest scorable concentration used in the test. The percentage of cells in metaphase per at least 1000 cells scored per concentration level (at least 500 from each duplicate culture) was determined prior to coding the slides. After selection of the slides for cytogenetic analyses, the slides were coded and scored. Metaphase cells were selected for scoring based on good chromosome morphology and staining characteristics. Only metaphase cells with 20 ± 2 centromeres were analyzed for structural aberrations. At least 200 metaphases per concentration level (100 from each duplicate culture), when available, were analyzed for structural aberrations.[(5)] Numerical aberrations were recorded as well. The number of metaphases evaluated per duplicate flask was less if 10 or more aberrant cells were observed among the first 25 cells scored. Chromatid-type aberrations included chromatid and isochromatid breaks and exchange figures. Chromosome-type aberrations included chromosome breaks and exchange figures. Pulverized chromosome(s) and cells, and severely damaged cells (i.e., cells with

≥10 aberrations per cell) were recorded and included in the analyses. The XY coordinates for the microscope stage were recorded for cells with structural aberrations.

7. Chromosome Aberration Assay

Target concentrations for the chromosome aberration assay were achieved in the preliminary toxicity assay; selected slides were coded and evaluated for clastogenicity. No separate chromosome aberration assay was conducted.

E. Criteria for Determination of a Valid Test

An assay was considered acceptable for evaluation of test results only if all of the following criteria were satisfied. The metabolically activated and non-activated assays of the test are independent and, if necessary, were repeated separately.

1. Negative Controls

The frequency of cells with structural chromosome aberrations was in the frequency range of the historical control vehicle.

2. Positive Controls

The percentage of cells with structural chromosome aberrations must be statistically significantly greater ($p < 0.05$, Fisher's exact test) than the vehicle control response.

F. Evaluation of Test Results and Statistical Analyses

The clastogenic potential of the test substance was assessed based on its ability to induce structural chromosome aberrations. The experimental unit is the cell; therefore the percentage of cells with structural aberrations was used for the assessment.

Data was evaluated using scientific judgment. Statistical analysis was used as a guide to determine whether or not the test substance induced a positive response. Interpretation of the statistical analysis also relied on additional considerations including the magnitude of the observed test substance response relative to the vehicle control response and the presence of a dose responsive trend. Statistical analysis consisted of a Cochran-Armitage test for dose responsiveness and a Fisher's exact test to compare the percentage of cells with structural or numerical aberrations (or the percentage of cells with more than one aberration, if required) in the test substance treated groups with the vehicle control response.[6-7] At the discretion of the study director, statistical analyses may be conducted on the percentage of cells with numerical aberrations as well.

The following conditions were used as a guide to determine a positive response:

- A statistically significant increase ($p < 0.05$, Fisher's exact test) in the percentage of cells with structural aberrations was seen in one or more treatment groups relative to the vehicle control response.

- The observed increased frequencies were accompanied by a concentration-related increase.
- A statistically significant increase was observed at the highest dose only.
- Note: Statistically significant values that did not exceed the historical control range for the negative/vehicle control may be judged as not being biologically significant.

The following condition was used as a guide to determine an equivocal response:

- Results observed in any of the assays resulted in statistically significant elevations in structural chromosome aberrations at more than one test concentration level, except the highest dose, without demonstrating a dose-responsive trend.

The test substance was judged negative if the following condition was met:

- There was no statistically significant increase in the percentage of cells with structural aberrations in any treatment group relative to the vehicle control group.

G. Data Presentation

The data are summarized in tables containing cell counts, cell growth inhibition, mitotic index, number of cells analyzed, types of structural aberrations, frequencies of structural aberrations per cell, and the percentage of cells with structural or numerical (polyploidy/endoreduplication) aberrations. Chromatid and chromosome gaps are listed but were not added to the totals for structural aberration evaluation (gaps are not considered true structural damage).

RESULTS AND DISCUSSION

A. Solubility

Water was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in the vehicle at the highest stock concentration prepared, 34.71 mg/mL. Due to the discrepancy between the initial sponsor-reported and COA-reported purities, the actual stock concentration that was prepared was 33.91 mg/mL. No visible precipitate was observed in the treatment medium at any concentration at the beginning or end of the treatment periods in the assay.

B. Preliminary Toxicity Assay

(Table 1-7)

CHO cells were exposed to a total of seven concentrations of the test substance ranging from 50 to 3471 µg/mL (10 mM), as well as positive controls and a vehicle control. Due to the discrepancy between the initial sponsor-reported and COA-reported purities, the actual maximum exposure concentration was 3391 µg/mL. The lower concentration levels were consequently reduced by 2.3% each. All subsequent concentration levels in this report represent the nominal values based on the initial sponsor-reported purity (84.5%) used for study conduct. The cells were exposed for 4 hours in both the absence and presence of an exogenous metabolic activation system (Aroclor-induced S9), or for 20 hours in the absence of S9 activation. The test substance was soluble in the treatment medium at all concentrations tested. No precipitation was observed at the beginning or end of the treatment periods at any concentration. Osmolality and pH measurements were taken from the highest test substance concentration (3471 µg/mL) and the vehicle control media. Based on visual inspection of the pH-sensitive treatment medium at the beginning and end of the treatment periods, the pH of the highest as well as the 2000 µg/mL test substance concentrations in media was higher than the pH of the vehicle control. However, when the pH was measured at the beginning of the exposure periods, the values were similar for the vehicle controls and the highest test substance concentration. For example, in the non-activated test system, the measured pH for the highest test substance concentration in media was 8.12 compared to 7.90 for the vehicle control. In the S9-activated test system, the measured pH for the highest test substance concentration in media was 7.85 compared to 7.61 for the vehicle control. The osmolality of the highest test substance concentration tested in treatment media was 289 and 299 mmol/kg in the non-activated and activated test condition, respectively. The osmolality of the vehicle in the treatment media was 265 and 273 mmol/kg in the non-activated and activated test condition, respectively. The observed changes in osmolality were ≤20% and were not considered significant. The cultures were microscopically inspected for the extent of monolayer confluence relative to the vehicle control. These data are not included in the report; the assessment was mainly an aid for study conduct. In addition, the uncoded slides were microscopically observed for the presence of mitotic cells and the mitotic cells were counted to assess a mitotic index to ensure the selection of scorable test substance concentrations (Tables 2-4). Substantial toxicity (greater than a 50% reduction in cell growth relative to the vehicle control) was observed at 3471 µg/mL in the 20-hour non-activated test condition only. The mitotic inhibition in the 20-hour non-activated test condition at 3471 µg/mL was 96%. Target concentrations for the chromosome aberration assay were achieved in the preliminary toxicity assay. Slides were coded and evaluated for clastogenicity and no separate chromosome aberration assay was conducted. Selection of doses for microscopic analysis was therefore based on these dose concentration levels.

The cytogenetic analysis findings from the individual treatment cultures in the non-activated 4-hour exposure group are presented in Table 2 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 3471 µg/mL, a 32% growth inhibition in relation to the vehicle control was observed (Table 1). The mitotic inhibition was 34.6% relative to the vehicle control. The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control group at any concentration ($p \geq 0.05$, Fisher's exact test). The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (42%) was statistically significant ($p < 0.05$, Fisher's exact test).

The cytogenetic analysis findings from the individual treatment cultures in the S9-activated 4-hour exposure group are presented in Table 3 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 3471 µg/mL, a 17% growth inhibition in relation to the vehicle control was observed (Table 1). The mitotic inhibition was 36.7% relative to the vehicle control. The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control group at any concentration ($p \geq 0.05$, Fisher's exact test). However, the control value for numerical aberrations was high (outside of the historical control range). The percentage of cells with structurally damaged chromosomes in the CP (positive control) treatment group (26.5%) was statistically significant ($p < 0.05$, Fisher's exact test).

The cytogenetic analysis findings from the individual treatment cultures in the non-activated 20-hour exposure group are presented in Table 6 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 2000 µg/mL, a 41% growth inhibition in relation to the vehicle control was observed (Table 1). The mitotic inhibition was 80.1% relative to the vehicle control. The percentage of cells with numerical aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control group at 500 µg/mL only ($p \leq 0.05$, Fisher's exact test). This response was not dose-dependent and is not considered biologically significant. The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (42%) was statistically significant ($p < 0.05$, Fisher's exact test).

To confirm the results obtained in the 4-hour activated test condition in trial 1 (preliminary toxicity assay), a confirmatory trial (trial 2) was conducted at concentration levels of 250, 500, 1000, 2000, and 3471 µg/mL for this test condition. No visible precipitate was observed in the treatment medium at the beginning or end of the treatment period at any concentration tested. No substantial toxicity was observed at any concentration level. The cytogenetic analysis findings from the individual treatment cultures are presented in Table 5 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 3471 µg/mL, a 37% growth inhibition in relation to the vehicle control was observed (Table 4). The mitotic inhibition was 14% relative to the vehicle control. The percentage of cells with structural aberrations was significantly increased above that of the vehicle control at

3471 µg/mL ($p < 0.05$, Fisher's exact test). The response was outside the historical control range and is considered biologically significant. The percentage of cells with structurally damaged chromosomes in the CP (positive control) treatment group (32.5%) was statistically significant ($p < 0.05$, Fisher's exact test).

CONCLUSION

All criteria for a valid study were met. Under the conditions of this study, H-28072 was found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells in the S9-activated test system. It was concluded that the test substance was positive in this *in vitro* test.

RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, the protocol, amendments (if any), and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

REFERENCES

1. Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., and Wassom, J.S. (1981). Mammalian in vivo and in vitro cytogenetic assays: A report of the Gene-Tox Program, Mutation Research, 87:143-188.

2. Evans, H.J. (1976). Cytological Methods for Detecting Chemical Mutagens. In: Chemical Mutagens, Principles and Methods for their Detection, Vol. 4, Hollaender, A. (ed.), Plenum Press, New York and London.

3. Swierenga, S.H.H., Heddle, J.A., Sigal, E.A., Gilman, J.P.W., Brillinger, R.L., Douglas, G.R., and Nestmann, E.R. (1991). Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese lung and human lymphocyte cultures, Mutation Research 246:301-322.

4. Galloway, S.M., Aardema, M.J., Ishidate Jr., M., Ivett, J.L., Kirkland, D.J., Morita, T., Mosesso, P., and Sofuni, T. (1994). Report from working group on in vitro tests for chromosomal aberrations, Mutation Research 312(3):241-261.

5. Scott, D., Danford, N.D., Dean, B.J., and Kirkland, D.J. (1990). Metaphase Chromosome Aberration Assays In Vitro. In: Basic Mutagenicity Tests: UKEMS Recommended Procedures. D.J Kirkland (ed). Cambridge University Press, New York, NY.

6. Fisher, R.A. (1985). Statistical Methods for Research Workers, 13th edition. Haffner, New York.

7. Snedecor, G.W. and Cochran, W.G. (1967). Statistical Methods, 6th edition, pp 246-248 and 349-352. The Iowa State University Press, Iowa.

TABLES

TABLES

EXPLANATORY NOTES

ABBREVIATIONS:

% Aberrant Cells	Cells with numerical aberrations include polyploid and endoreduplicated cells; cells with structural aberrations exclude cells with only gaps
Aberrations Per Cell	Cells with severely damaged chromosomes or with 10 or more structural aberrations were counted as 10 aberrations
Br	break
Cell Growth Index	(cells per flask treated group/cells per flask control group), expressed as a percentage
Cell Growth Inhibition	(cell growth index control group – cell growth index treated group)
CHO	Chinese hamster ovary
Chromatid Aberrations	include chromatid and isochromatid breaks and fragments (Br); chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements
Chromosome Aberrations	include breaks and acentric fragments (Br); Dic, dicentric chromosome
Ex	exchange
Mitotic Index	(cells in mitosis / # cells scored), expressed as a percentage
SD	standard deviation
Severely Damaged Cells	includes cells with one or more pulverized chromosome and cells with 10 or more structural aberrations

NOTES:
All calculated values are rounded.

Table 1
Preliminary toxicity test using H-28072 in the absence or presence of exogenous metabolic activation

		4 Hours						20 Hours		
		S9-			S9+			S9-		
Treatment[a] (µg/mL)	Flask	Cell Count (cells/mL x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition[b] (%)	Cell Count (cells/mL x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (cells/mL x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)
Vehicle[c]	A B	0.53 0.45	100	NA	0.48 0.53	100	NA	0.46 0.47	100	NA
50	A B	0.44 0.51	97	3	0.52 0.49	100	0	0.52 0.52	112	-12
100	A B	0.52 0.49	103	-3	0.47 0.44	90	10	0.52 0.47	106	-6
250	A B	0.46 0.52	100	0	0.43 0.41	83	17	0.41 0.50	98	2
500	A B	0.47 0.44	93	7	0.47 0.45	91	9	0.43 0.37	86	14
1000	A B	0.49 0.48	99	1	0.49 0.46	94	6	0.29 0.30	63	37
2000	A B	0.45 0.45	92	8	0.47 0.44	90	10	0.23 0.32	59	41
3471[d]	A B	0.31 0.36	68	32	0.42 0.42	83	17	0.15 0.17	34	66
Low PC[e]	A B	0.40 0.37	79	21	0.32 0.36	67	33	0.29 0.29	62	38
High PC[f]	A B	0.32 0.34	67	33	0.26 0.28	53	47	0.35 0.30	70	30

[a]CHO cells were treated at 37ºC.
[b]Negative value indicates growth increase and therefore no growth inhibition.
[c]Water
[d]Equivalent to a 10 mM concentration.
[e]Low PC: 0.2 µg/mL MMC for non-activated test systems; 5 µg/mL CP for S9-activated test systems.
[f]High PC: 0.4 µg/mL MMC for non-activated test systems; 10 µg/mL CP for S9-activated test systems.

Table 2
Cytogenetic analysis of CHO cells treated with H-28072 in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Flask	Mitotic Index (%)	Cell Scored Numerical	Cell Scored Structural	% Aberrant Cells[b] Numerical	% Aberrant Cells[b] Structural	Total Number of Structural Aberrations: Gaps	Chromatid Br	Chromatid Ex	Chromosome Br	Chromosome Ex	Severely Damaged Cells	Average Aberrations Per Cell
Vehicle[c]	A	13.8	100	100	3	2	1	2	0	0	0	0	0.020
	B	12.8	100	100	4	2	2	1	0	1	0	0	0.020
1000	A	10.0	100	100	5	0	2	0	0	0	0	0	0.000
	B	9.8	100	100	2	4	4	3	0	0	1	0	0.040
2000	A	11.6	100	100	7	3	0	3	0	0	0	0	0.030
	B	10.2	100	100	6	4	2	3	0	1	0	0	0.040
3471[d]	A	8.2	100	100	6	1	0	1	0	0	0	0	0.010
	B	9.2	100	100	11	0	1	0	0	0	0	0	0.000
MMC 0.2	A	10.4	25	25	8	44	0	7	3	1	0	0	0.440
	B	7.6	25	25	4	40	0	11	3	1	0	0	0.600

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 3
Cytogenetic analysis of CHO cells treated with H-28072 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a]		Mitotic	Cell Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely	Average
		Index						Chromatid		Chromosome		Damaged	Aberrations
(µg/mL)	Slide	(%)	Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex	Cells	Per Cell
Vehicle[c]	A	14.8	100	100	11	2	1	1	0	1	0	0	0.020
	B	16.8	100	100	7	4	2	3	0	1	0	0	0.040
1000	A	10.8	100	100	5	1	2	0	0	1	0	0	0.010
	B	14.6	100	100	5	3	0	3	0	0	0	0	0.030
2000	A	13.6	100	100	6	2	0	1	0	0	1	0	0.020
	B	9.6	100	100	7	1	2	1	0	0	0	0	0.010
3471[d]	A	9.8	100	100	13	2	1	1	3	1	0	0	0.050
	B	10.2	100	100	10	3	3	1	2	0	0	0	0.030
CP 5	A	7.0	100	100	3	9	2	7	3	0	0	0	0.100
	B	7.8	25	25	4	44	1	7	6	2	1	0	0.640

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 4
Concurrent toxicity test using H-28072 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period) - TRIAL 2

Treatment[a] (µg/mL)	Flask	Cell Count (cells/mL x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)	Mitotic Index (%)	Mitotic Inhibition[b] (%)
Vehicle[c]	A B	0.68 0.61	100	NA	15.0	NA
250	A B	0.60 0.58	91	9	NA	NA
500	A B	0.57 0.58	89	11	NA	NA
1000	A B	0.54 0.62	90	10	13.1	12.7
2000	A B	0.51 0.53	81	19	10.4	30.7
3471[d]	A B	0.42 0.39	63	37	12.9	14.0
CP 5	A B	0.37 0.33	54	46	8.6	42.7
CP 10	A B	0.31 0.35	51	49	5.2	65.3

[a]CHO cells were treated at 37ºC.
[b]Negative value indicates mitotic increase and therefore no mitotic inhibition.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 5
Cytogenetic analysis of CHO cells treated with H-28072 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period) - TRIAL 2

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cell Scored Numerical	Cell Scored Structural	% Aberrant Cells[b] Numerical	% Aberrant Cells[b] Structural	Total Number of Structural Aberrations: Gaps	Chromatid Br	Chromatid Ex	Chromosome Br	Chromosome Ex	Severely Damaged Cells	Average Aberrations Per Cell
Vehicle[c]	A	16.4	100	100	4	3	2	3	0	0	0	0	0.030
	B	13.6	100	100	1	0	1	0	0	0	0	0	0.000
1000	A	12.6	100	100	3	2	1	3	0	0	1	0	0.040
	B	13.6	100	100	1	3	0	4	0	0	0	0	0.040
2000	A	10.2	100	100	4	3	0	3	0	0	2	0	0.050
	B	10.6	100	100	4	2	1	2	0	0	0	0	0.020
3471[d]	A	12.0	100	100	7	7	3	6	1	1	0	0	0.080
	B	13.8	100	100	9	7	3	6	2	0	2	0	0.100
CP 5	A	7.4	25	25	0	44	1	14	2	2	0	0	0.720
	B	9.8	100	100	3	21	2	7	12	3	1	0	0.230

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 6
Cytogenetic analysis of CHO cells treated with H-28072 in the absence of exogenous metabolic activation (20-hour continuous treatment)

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cell Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	16.4	100	100	0	3	0	2	0	1	0	0	0.030
	B	13.8	100	100	3	1	2	1	0	0	0	0	0.010
500	A	8.8	100	100	7	3	2	1	0	1	1	0	0.030
	B	7.0	100	100	7	5	3	4	0	0	1	0	0.050
1000	A	6.2	100	100	3	2	3	2	0	0	0	0	0.020
	B	5.0	100	100	7	4	2	5	0	0	0	0	0.050
2000[d]	A	3.6	100	100	4	2	1	2	0	0	0	0	0.020
	B	2.4	100	100	6	2	0	2	0	0	0	0	0.020
MMC 0.2	A	6.4	25	25	4	40	0	4	4	2	0	0	0.400
	B	5.2	25	25	4	44	0	6	5	2	0	0	0.520

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 5.76 mM concentration.

Table 7
Summary

Treatment[a] µg/mL	S9 Activation	Treatment Time	Mitotic Index (%)	Cells Scored Numerical	Cells Scored Structural	Aberrations Per Cell Mean	Aberrations Per Cell SD	Cells with Aberrations[b] Numerical (%)	Cells with Aberrations[b] Structural (%)	
Vehicle[c]	-S9	4	13.3	200	200	0.020	0.000	3.5	2.0	
1000	-S9	4	9.9	200	200	0.020	0.028	3.5	2.0	
2000	-S9	4	10.9	200	200	0.035	0.007	6.5	3.5	
3471[d]	-S9	4	8.7	200	200	0.005	0.007	8.5	0.5	
MMC 0.2	-S9	4	9.0	50	50	0.520	0.113	6.0	42.0[f]	
Vehicle	+S9	4	15.8	200	200	0.030	0.014	9.0	3.0	
1000	+S9	4	12.7	200	200	0.020	0.014	5.0	2.0	
2000	+S9	4	11.6	200	200	0.015	0.007	6.5	1.5	
3471	+S9	4	10.0	200	200	0.040	0.014	11.5	2.5	
CP 5	+S9	4	7.4	125	125	0.370	0.382	3.5	26.5[f]	
Vehicle	-S9	20	15.1	200	200	0.020	0.014	1.5	2.0	
500	-S9	20	7.9	200	200	0.040	0.014	7.0	4.0	
1000	-S9	20	5.6	200	200	0.035	0.021	5.0	3.0	
2000[e]	-S9	20	3.0	200	200	0.020	0.000	5.0	2.0	
MMC 0.2	-S9	20	5.8	50	50	0.460	0.085	4.0	42.0[f]	
Vehicle	+S9	4	15.0	200	200	0.015	0.021	2.5	1.5	confirmatory
1000	+S9	4	13.1	200	200	0.040	0.000	2.0	2.5	confirmatory
2000	+S9	4	10.4	200	200	0.035	0.021	4.0	2.5	confirmatory
3471	+S9	4	12.9	200	200	0.090	0.014	8.0	7.0[f,g]	confirmatory
CP 5	+S9	4	8.6	125	125	0.475	0.346	1.5	32.5[f]	confirmatory

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.
[e]Equivalent to a 5.76 mM concentration.
[f]Statistically significant difference from control at $p < 0.05$ by Fisher's exact test.
[g]Statistically significant difference from control at $p < 0.05$ by Cochran-Armitage trend test.

APPENDICES

Appendix A
Certificate of Analysis

E. I. du Pont de Nemours and Company
Wilmington, DE 19898
USA

CERTIFICATE OF ANALYSIS

This Certificate of Analysis fulfills the requirement for characterization of a test substance prior to a study subject to GLP regulations. It documents the identity and content of the test substance. This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 792).

Haskell Code Number	H-28072
Common Name	HFPO Dimer Acid Ammonium Salt
Purity Percent	82.6%
Other Components	Water – 13.9% Ammonium (excess) – 3.5%
Date of Analysis	July 19, 2007
Recommended reanalysis interval	1 year
Instructions for storage	NRT&H
Reference	DuPont-23285
Analysis performed at	E. I. DuPont de Nemours and Company DuPont Haskell Laboratories Newark, Delaware USA

Peter A. Bloxham, Ph.D.		25-JULY-2007
Analyst's Name	Analyst's signature	Date

Revision #1
July 20, 2007

Appendix B
Historical Control Data

HISTORICAL CONTROL DATA[a]

Historical Values	Non-Activated Test System: Solvent Control (%)	Non-Activated Test System: Positive Control[b] (%)	S9-Activated Test System: Solvent Control (%)	S9-Activated Test System: Positive Control[c] (%)
Structural Chromosome Aberrations				
Mean	1.10	37.6	1.45	42.7
Standard Deviation	1.32	15.3	1.86	17.0
Range	0 – 5	10 – 62	0 – 6	9.5 – 68
Numerical Chromosome Aberrations				
Mean	1.41	0.77	1.32	0.40
Standard Deviation	1.84	1.68	1.60	0.44
Range	0 – 5	0 – 4	0 – 5	0 – 2

a Data are based on studies conducted 2003-2006. Data include all control solvents or diluents and metabolic activation systems based on Aroclor-induced rat liver S9.
b Mitomycin C (MMC)
c Cyclophosphamide (CP)